526 Rec'd PCT/PTO 28 NOV 2000

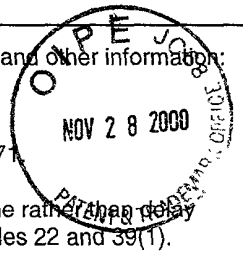
FORM PTO-1390 (REV 11-98)	U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 124-809
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U S. APPLICATION NO (If known, see 37 C.F R. 1.5) 09/701299 (To Be Assigned)
INTERNATIONAL APPLICATION NO. PCT/GB99/01387	INTERNATIONAL FILING DATE 5 May 1999	PRIORITY DATE CLAIMED 29 May 1998

TITLE OF INVENTION

RECOMBINANT VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE

APPLICANT(S) FOR DO/EO/US

BENNETT



Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☒ has been transmitted by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   - d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information. **PTO-1449/ International Search Report**

☐ This application is entitled to "Small entity" status. ☐ "Small entity" statement attached.

478192

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U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
(To Be Assigned) 09/720129	PCT/GB99/01387	124-809

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):

-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....$860.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2) paid to USPTO $710.00

-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....$690.00

-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =						$	860.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).						$	0.00
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE			
Total Claims	18	-20 =	0	X	$18.00	$	0.00
Independent Claims	2	-3 =	0	X	$80.00		0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					$270.00	$	0.00
TOTAL OF ABOVE CALCULATIONS =						$	**860.00**
Reduction by ½ for filing by small entity, if applicable. Small entity status must also be asserted. (Note 37 C.F.R. 1.9, 1.27, 1.28).							0.00
SUBTOTAL =						$	**860.00**
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +							0.00
TOTAL NATIONAL FEE =						$	**860.00**
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +						$	40.00
Fee for Petition to Revive Unintentionally Abandoned Application ($1240.00 – Small Entity = $620.00)						$	0.00
TOTAL FEES ENCLOSED =						$	**900.00**
						Amount to be: refunded	$
						Charged	$

a. ☒ A check in the amount of $900.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.

d. ☐ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201
Telephone: (703) 816-4000

SIGNATURE

Arthur R. Crawford
NAME

25,327 **November 28, 2000**
REGISTRATION NUMBER Date

478192

#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

BENNETT

Serial No. **09/701,299**

Filed: **November 28, 2000**

Atty. Ref.: **124-809**

Group:

Examiner:

For: **RECOMBINANT VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE**

* * * * * * * * * * * *

June 29, 2001

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

AMENDMENT

Responsive to the Notification dated April 30, 2001, entry and consideration of the following amendments and remarks are requested.

IN THE SPECIFICATION

Amend the specification as follows.

"Primer 1 designated " Nsi 1 ""

and insert the following new paragraph therefor:

--Primer 1 designated "Nsi 1" (SEQ ID NO:4)--.

Page 9, delete the following paragraph on line 25

"Primer 2 designated "Nsi 2""

and insert the following new paragraph therefor:

--Primer 2 designated "Nsi 2" (SEQ ID NO:5)--.

Page 10, delete the following paragraph at line 21

"Oligo 1 designated "7.5KF2""

and insert the following new paragraph therefor:

--Oligo 1 designated "7.5KF2" (SEQ ID NO:6)--.

Insert the attached Sequence Listing after the claims.

REMARKS

The specification has been amended to include sequence identifiers and the attached Sequence Listing, in response to the Notification dated April 30, 2001 (copy attached). No new matter has been added. The attached paper and computer-readable copies of the Sequence Listing are the same. A separate Letter to this effect is attached.

An early and favorable Action on the merits is requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

B. J. Sadoff
Reg. No. **36,663**

BJS:eaw
1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

MARKED-UP SPECIFICATION

Page 9, delete the following paragraph at line 21

"Primer 1 designated " Nsi 1 ""

and insert the following new paragraph therefor:

--Primer 1 designated "Nsi 1" (SEQ ID NO:4)--.

Page 9, delete the following paragraph on line 25

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and insert the following new paragraph therefor:

--Oligo 1 designated "7.5KF2" (SEQ ID NO:6)--.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

BENNETT Atty. Ref.: **124-809**

Serial No. **(To Be Assigned)** Group:

National Phase of **PCT/GB99/01387**

Filed: **November 28, 2000** Examiner:

For: **RECOMBINANT VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE**

* * * * * * * * * * *

November 28, 2000

Assistant Commissioner for Patents
Washington, DC 20231
Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend the claims as follows:

IN THE CLAIMS

Claim 5, line 1, delete "or claim 4".

Claim 9, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 10, line 1, change "any one of the preceding claims" to --claim 1--.

Claim 12, line 1, delete "or claim 11".

Claim 15, line 2, change "any one of the preceding claims" to --claim 1--.

Claim 16, lines 3-4, change "any one of claims 1 to 14" to --claim 1--.

Claim 18, line 2, change "any one of claims 1 to 14" to --claim 1--.

REMARKS

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

Arthur R. Crawford
Reg. No. **25,327**

ARC:ms

1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

RECOMBINANT VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE

The present invention relates to a virus vaccine, specifically a vaccine to Venezuelan equine encephalomyelitis virus (VEE), to its preparation and pharmaceutically acceptable formulations and methods of prophylactic and therapeutic methods of treatment using said vaccine.

VEE virus is a mosquito-borne alphavirus which is an important cause of epidemic disease in humans and of epizootics in horses, donkeys and mules in certain parts of the world, in particular the South Americas.

The existing VEE vaccine, TC-83, was initially produced by attenuation of the Trinidad donkey strain (TRD) of VEE by sequential passage in guinea pig heart cell cultures. However, this vaccine is generally regarded as being inadequate for human vaccination. This is mainly due to the high incidence of side effects in vaccinees and the large proportion of vaccinees who fail to develop neutralising antibodies (Monath et al. 1992, Vaccine Research, **1**, 55-68).

A vaccinia-based vaccine against VEE has been constructed (Kinney et al. J. Gen. Virol. 1988, 69, 3005-3013). In this recombinant, 26S RNA encoding structural genes of VEE were inserted into the NYCBH strain of vaccinia. The recombinant virus protected against sub-cutaneous challenge but had limited efficacy against aerosol challenge with VEE.

The virulent Trinidad donkey strain of VEE and the attenuated strain TC-83 have both been cloned and sequenced (R.M. Kinney et al. Virology (1989) **170**, 19-30) and the amino acid and nucleotide numbering system used in this reference will be used hereinafter. This work has revealed that there are a number of amino acid changes between TRD and TC-83. The majority (five) of these changes occur within the gene encoding the glycoprotein E2.

The changes have been summarised as follows:

Table 1

	Nucleotide change		Amino acid	
Position	TRD	TC-83	TRD	TC-83
22, junction region	A	G	non-coding	
1053, E_2-7	G	U	Lys	Asn
1285, E_2-85	C	U	His	Tyr
1391, E_2-120	C	U	Thr	Arg
1607, E_2-192	U	A	Val	Asp
1866, E_2-278	U	C	none	
1919, E_2-296	C	U	Thr	Ile
2947, E_1-161	U	A	Leu	Ile
3099, E_1-211	A	U	none	
3874, 3'-non-coding region	UU	U	non-coding	

It has also been shown that the first 25 amino acids of the E2 glycoprotein represents a protective epitope. This region includes a single amino acid change (lys → asp) at amino acid 7 in the TC-83 construct as compared to the TRD strain. A 25bp synthetic peptide based on the TRD sequence VE2pep01(TRD), protected more mice from TRD virus challenge than a corresponding TC-83 based peptide (A.R. Hunt et al., Virology, 1990, 179, 701-711). More precise mapping of this epitope has been carried out (A.R Hunt et al., Vaccine 1995, **13**, 3, 281-288).

The applicants have found ways of increasing the protectiveness of a vaccine and in particular a vaccinia-based vaccine.

In particular, the applicants have found that the protectiveness of the vaccine may be increased either (a) by restoring the lysine residue at amino acid 7 of the E2 protein and/or (b) by modifying the promoter to increase expression of the protective construct.

Thus, in a first aspect, the present invention provides a vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine

comprising a vector which includes a sequence which encodes an attenuated form of said virus which is capable of producing a protective immune response, wherein the said sequence is such that the amino acid at position 7 in the E2 protein of VEE is lysine.

Suitably, the attenuated form of the VEE virus comprises a derivative or variant of the TC-83 construct or an immunogenic fragment thereof.

Other attenuated forms may be produced by the skilled person, for example using known techniques such as serial passage through another organism, or by recombinant DNA technology, for instance by inactivating genes associated with the replication or virulence of the virus. The structural gene encoding the E2 glycoprotein or a fragment encoding at least the N-terminal 19 amino acids should be retained in order to retain immunogenicity of the construct.

Suitable fragments of the construct are those which include only some of the structural genes of the VEE peptide or which encode only part of the proteins encoded by said genes, provided the construct encodes sufficient antigenic determinants to ensure that it is capable of producing a protective immune response in a mammal to whom the construct is administered.

As used herein, the term " variant" means that the construct is different to the original strain but that it encodes proteins and/or peptides which are the same or similar to those of wild-type VEE or immunogenic fragments thereof. Thus, the changes in the nucleotide sequence may be silent in that they do not produce amino acid changes as compared to the original strain, or they may produce amino acid changes provided these do not alter function of the construct in terms of its ability to produce a protective immune response against VEE. For example, the construct may encode peptides or proteins which are 60% homologous to the wild-type proteins or peptides, suitably more than 80% homologous and preferably more than 90% homologous to the native protein sequence, and provided they produce antibodies which are cross-reactive with wild-type VEE, the protective effects of the construct may be retained.

" Derivatives" may have broadly similar structures but they are derived by manipulating the original constructs using recombinant DNA technology or chemical modification if appropriate.

The vector may contain the usual expression control functions such as promoters, enhancers and signal sequences, as well as a selection marker in order to allow detection of successful transformants. The selection of these will depend upon the precise nature of the vector chosen and will be known to or readily determinable by a person skilled in the art.

Suitably the vector is a viral vector, for example a vector derived from *vaccinia*, *adenovirus*, or herpes simplex virus (HSV) BCG or BCC. It is suitably attenuated itself, to minimise any harmful effects associated with the virus on the host.

Preferably, the vector is derived from vaccinia virus, as it has many properties which make it a suitable vector for vaccination, including its ability to efficiently stimulate humoral as well as cell-mediated immune responses. Vaccinia has proven utility as a vaccine vehicle, following the Smallpox eradication programmes. It provides the potential for multi-valent vaccine construction and for oral administration. There are many attenuated strains currently available.

A suitable selection marker for inclusion in a vaccinia vector is the *gpt* marker gene.

A VEE vaccine was constructed using a WR strain of vaccinia in this work. Preferably, a more highly attenuated strain of vaccinia which would be more acceptable for use in humans is employed. Such strains include Lister, which was used for wide scale vaccination against smallpox, NYVAC (Tartaglia *et al*, (1992). *AIDS Research and Human Retroviruses* 8,1445-1447) which contains specific genome deletions, or MVA (Mayr *et al*, (1975) *Infection* 3, 6-14) which is also highly attenuated.

Vaccines based upon viral vectors are suitably formulated for parenteral administration as described above. However, it is possible to formulate such vaccines for oral administration, for

example by incorporating the vector into a gut-colonising microorganism such as *Salmonella* and particularly *S. typhimurium*.

pTC-5A is a plasmid clone of cDNA encoding the structural genes of VEE virus strain TC-83 (Kinney et al. J. Gen. Virol. (1988) **69**, 3005-30130). The VEE cDNA is situated downstream of the vaccinia 7.5K promoter which drives expression of the VEE structural proteins when the plasmid is used to construct recombinant vaccinia viruses. Modified 7.5K vaccinia promoters have previously been prepared (Davison & Moss, J. Mol. Biol. **210**, (1989) 749-769). It has been found that certain substitution mutations increase the strength of the promoter. By using synthetic promoters which include substitution mutations, the amount of VEE proteins produced from the recombinant virus was increased.

Thus in a further aspect of the invention, there is provided a vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine comprising a vaccinia virus vector which encodes an attenuated form of the VEE virus or a variant or fragment thereof which is capable of producing a protective immune response against VEE virus, expression of the said attenuated VEE virus being under the control of a synthetic 7.5K vaccinia promoter which has been subject to mutation which increases the level of VEE virus protein production as compared to the wild-type 7.5K promoter.

In particular, it has been found that substitution mutations within the 7.5Kd promoter can be effective. These may be illustrated by the following Table:

Wild-type 7.5K promoter:

TAAAAGTAGAAAATATATTCTAATTTATTGCAC (SEQ ID No 1)

Substitution Mutations (emboldened)

TAAAAA**TT**GAAAAT**AC**ATTCTAATTTATTGCAC (SEQ ID No 2)

TAAAAA**TT**GAAAAT**AT**ATTCTAATTTATTGCAC (SEQ ID No 3)

Inclusion of a synthetic 7.5K vaccinia promoter in WR103 has

been found to increase expression of the downstream VEE cDNA, leading to a 3.59-fold increase in protein production.

The vaccine may comprise the vector itself but it is suitably formulated as a pharmaceutical composition in combination with a pharmaceutically acceptable carrier or excipient. Such compositions form a further aspect of the invention. The compositions may be in a form suitable for oral or parenteral application.

Suitable carriers are well known in the art and include solid and liquid diluents, for example, water, saline or aqueous ethanol. The liquid carrier is suitably sterile and pyrogen free.

The compositions may be in the form of liquids suitable for infusion or injection, or syrups, suspensions or solutions, as well as solid forms such as capsules, tablets, or reconstitutable powders.

Constructs for use in the vaccines of the invention may be prepared by various means as will be understood in the art, ranging from modification of available constructs such as the wild-type virus using recombinant DNA technology or by synthetic means. Recombinant DNA techniques include site directed mutagenesis, optionally involving PCR amplification as illustrated hereinafter.

As illustrated hereinafter, recombinant vaccinia virus was constructed which expressed the structural genes of VEE as produced by a modified form of TC-83. The ability of the recombinant virus to elicit protective immune responses against virulent VEE disease was investigated.

In yet another embodiment, the vaccine further comprises a cytokine or an active fragment or variant thereof. The cytokine may itself be incorporated into the vaccine formulation, or more suitably, the vector may include a coding sequence which means that the cytokine is co-expressed by the vector. Examples of suitable cytokines include interleukin 2 (IL-2) and interleukin 6 (IL-6).

A particularly suitable cytokine is interleukin 2 (IL-2), which may be expressed from for example a vaccinia virus recombinant. IL-2 is known to be responsible for the clonal expansion of antigen-activated T cells (Smith, (1984) *Reviews in Immunology* **2**, 319-333).

Alternatively, antibody levels can be enhanced using other cytokines. For example, expression of IL-6 by vaccinia vectors has been shown to induce a high level of IgG_1 (Ruby *et al*, 1992 *Vaccine Research* **1**, (4), 347-356), and IL-5 and IL-6 induced mucosal IgA responses to co-expressed influenza HA (Ramsay *et al*, (1994) Reproduction, Fertility and Development **6**, 389-392).

The vaccine of the present invention may be used to treat humans or animals. In particular it may be given to horses, as a veterinary vaccine, to prevent infection, or as a prophylactic or therapeutic vaccine for humans.

The vaccine of the invention may be incorporated into a multivalent vaccine in order to increase the benefit-to-risk ratio of vaccination.

The dosage of the vaccines of the invention will depend upon the nature of the mammal being immunised as well as the precise nature and form of the vaccine. This will be determined by the clinician responsible. However in general, when using a virus vector such as a vaccinia virus vectors, dosages of the vector may be in the range of from 10^4-10^{12}pfu (pfu = particle forming units).

The vaccines of the invention will produce an immune response in test animals including the production of antibodies. These antibodies may be useful in passive vaccination programmes or in diagnosis of VEE virus disease. For diagnostic purposes, the antibodies may form part of a kit as is conventional in the art.

The invention will now be illustrated by way of Example with reference to the accompanying drawings in which

Figure 1 shows the construction of chimeric plasmids used for

generation of recombinant vaccinia viruses;

Figure 2 shows the results of a immunofluoresence assay using polyclonal antiserum to TC-80;

Figure 3 is a graph showing the results of an experiment to quantify by ELISA the amount of VEE protein expressed by strains; and

Figure 4 is a graph showing the results of an experiment to find the level of anti-VEE IgG in animals vaccinated with various strains of the invention.

In the Examples, relative protein levels were calculated from ELISA data using regression analysis performed by Minitab statistical analysis software (Minitab Inc., State College, PA, USA). Serum antibody levels were compared by the two sample *t* test. Contingency tables were analysed by Fisher's exact test. *P* values of <0.05 were taken to be significant.

Example 1

Alteration of the E2 protein sequence

pTC-5A, a plasmid clone of cDNA encoding the structural genes of Venezuelan Equine Encephalitis virus, strain TC-83 was obtained from Dr. R, Kinney (Kinney et al, 1988, Journal of General Virology 69, 3005-3103). An *Eco* RI fragment containing the VEE cDNA was removed from pTC-5A and inserted into p1113 (Carroll, 1993, Ph.D. thesis, Faculty of Medicine, University of Manchester; Fig 1a) which is a shuttle vector used for insertion of genes into the thymidine kinase locus of vaccinia with dominant selection of recombinant viruses based on resistance to mycophenolic acid (Falkner & Moss, 1988, Journal of Virology 62, 1849-1854). The resulting plasmid, pAB100, was mixed with Lipofectin ™ (Life Technologies) and used to transfect CV-1 cells infected with vaccinia virus, strain WR. Recombinant viruses were designated WR100 and were subjected to three rounds of plaque-purification before preparation of stocks as described earlier (Mackett et al, 1985 DNA cloning (Volume II): a practical approach).

The sequence of VEE E2, strain TC-83, situated in pTC-5A, was

altered by one nucleotide substitution from T to G as position 1053 as compared to wild-type VEE TRD (Johnson et al. J. Gen. Virol. 1986, 67, 1951-1960). This resulted in an amino acid change from asparagine to lysine in the E2 protein when expressed from the vaccinia virus.

In order to perform this particular amino acid change, the following manipulations were carried out.

A cleavage site for restriction enzyme *Nsi I* occurs close to the site of the required nucleotide substitution. A second *Nsi I* site is situated about 500bp upstream. Oligonucleotide primers were used to amplify the DNA sequence between the *Nsi I* sites using the Polymerase Chain Reaction (PCR). The downstream primer contained a nucleotide mismatch corresponding to the TRD sequence at this point.

The primer sequences are listed below. The *Nsi I* cleavage sites and the position of the substituted nucleotide are underlined.

Primer 1 designated " Nsi 1"

5' GCC GAT GCA TGT GGA AGG C 3'

Primer 2 designated " Nsi 2"

5' ATC TGA TGC ATC TGG CCA TGT AAG GGC GCG TTA GCT TAT ACT CCT TAA ACA GC 3'

The PCR product was digested with *Nsi I* and used to replace the corresponding *Nsi I* fragment in pTC-5A, generating plasmid pAB101. The nucleotide sequence of the relevant region in pAB101 was obtained to verify the sequence alteration.

pAB101 was then digested with *Eco* R1 to remove the VEE 26S RNA coding sequence which was transferred to the vaccinia shuttle vector plasmid p1113. P1113 contains the selectable marker *gpt* which allows selection of recombinant vaccinia viruses. The plasmid constructed by the addition of the VEE sequence to p1113 was designated pAB102.

Example 2

Substitution of 7.5K promoter for a synthetic promoter in pAB102

A synthetic 7.5K vaccinia promoter was designed, based upon work by Davison and Moss (supra.). Complementary oligonucleotides were designed with 5'*Bam* HI and 3'*Eco* RI ends. The oligonucleotides were annealed and ligated into the plasmid pT7Blue (available from AMS Biotechnology (UK) Ltd). The plasmid clone was digested with *Bam* HI and *Eco* RI and the DNA fragment containing the synthetic promoter was isolated and cloned into the plasmid pAB102 which had been cut with the same enzymes. This resulted in the generation of plasmid pAB103 (Figure 1) which contains the synthetic promoter upstream of the VEE 26S RNA coding sequence. Vaccinia WR strain was transformed with PAB103 to produce the recombinant vaccinia virus WR103.

The sequence of the oligonucleotides used is given below. Substitutions in the 7.5K promoter sequence are given in bold type. Insertions are underlined. Oligonucleotide " tails" containing restriction enzyme cleavage sites are italicised.

Oligo 1 designated " 7.5KF2"

5' *ACG CGG ATC* CAA AAA TTG AAA AAC TAG CTT AAA AAT TGA AAA ACT ATT CTA ATT TAT TGC ACG *AAT TCC G* 3'

Oligo 2 designated " 7.5KR2"

This is the reverse complement of 7.5KF2.

The amount of VEE proteins produced by the recombinant virus WR103 was measured using enzyme linked immunoabsorbant assay (ELISA).

Example 3

Analysis of protein expression

VEE viral proteins were visualised by indirect immunofluorescence of infected CV-1 cells. CV-1 monolayers ($25cm^2$) were infected with virus at a multiplicity of 2 p.f.u. per cell. At 24 hours post infection, cells were scraped into the growth media and washed once with phophate-buffered saline (PBS) containing 0.1% bovine serum albumin. Cells were spotted onto slides, air-dried and fixed in acetone. Binding

of mouse polyclonal antiserum raised against VEE strain TC-80 (provided by Dr. A.D.T. Barrett, University of Texas) was detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Amersham International plc).

Examination of cells infected with WR100 or WR103 showed that WR103-infected cells fluoresced more brightly than the WR100-infected cells (Figure 2).

Quantification of VEE viral protein expression was carried out using an enzyme-linked immunosorbent assay (ELISA). CV-1 monolayers (150 cm^2) were infected with virus at a multiplicity of 10 p.f.u. per cell and harvested at 24 hours post infection by scraping into the growth media. Cells were washed once in PBS and resuspended in T9 buffer (10 mM Tris.HCl; 1 mM EDTA; pH 9.0). Samples were frozen, thawed and sonicated for 1 minute in a sonicating bath. Cells debris was pelleted for 5 minutes at 1800 g and the supernatant was centrifuged for 30 minutes at 10,000 x g. The supernatant was removed and stored at -70°C. The cell lysate preparation was diluted 1/30 in bicarbonate buffer (Sigma), 100 l volumes were added to wells of a microtitre plate and the antigen was allowed to bind at 37°C for 1 hour. Lysates were replaced with 200 l/well of saline containing 10% formaldehyde. Plates were incubated at room temperature for 20 minutes, then washed 6 times with PBS containing 0.1% Tween (PBST). Mouse polyclonal anti-TC80 was serially diluted in blocking solution (0.5% dried milk/PBST), added to wells, and the plates were incubated for 1 hour at 37°C. Plates were washed 3 times in PBST before addition of horseradish peroxidase-conjugated mouse specific antibody (diluted 1:1000 in blocking solution) and incubated for 1 hour at 37°C. Plates were washed 3 times before addition of ABTS in citrate buffer and incubation at room temperature for 1 hour. Colour development was measured at A_{414}.

This quantification process revealed that WR103-infected cells contained 3.59-fold more VEE protein than WR100-infected cells (Figure 3).

Quantification of vaccinia protein in these samples had demonstrated equivalent amounts in each (data not shown), so

it must be assumed that the difference in VEE protein content is due to different expression levels of the encoded VEE cDNA.

Example 4

Protective effect of Vaccinia recombinants

Groups (10) of female 6-8 week old Balb/c mice were inoculated with PBS or with 10^8 p.f.u. of vaccinia viruses by intramuscular injection, or with 10^5 p.f.u. of TC-83 by subcutaneous injection. Serum was taken for measurement of immunoglobulins to VEE proteins.

The vaccinated mice were challenged with two different doses of virulent VEE strain TRD at 35 days after immunisation. The survival rates after 14 days are presented in Table 2.

Table 2

Strain	10pfu TRD	100 pfu TRD
WR	0/10	0/10
WR100	1/10	2/10
WR103	6/10	6/10
No treatment	0/10	0/10

WR100: Vaccinia/VEE recombinant

WR103: Vaccinia/VEE recombinant produced in Example 2 above.

These results show that genetic manipulation of the recombinant virus has improved the protection afforded by the construct. A significant improvement in protection of mice following subcutaneous challenge with TrD was seen when WR103 was used for vaccination, compared with WR100 ($P<0.05$, Table 2). WR100 protected up to 20% of mice whereas WR103 protected 60% of mice. There was not a significant difference between numbers of mice protected when challenge doses of 10 p.f.u. or 100 p.f.u. of TrD were used. The challenge dose had previously been titrated to show that 1 p.f.u. of TrD approximates to 2-3 LD_{50} doses (data not shown).

Example 5

Immunoassays

VEE virus-specific immunoglobulin in serum was measured by enzyme-linked immunoassay as follows. Wells of a microtitre plate were coated with purified TC-83 at 37°C for 1 hour. Serum was diluted serially in blocking solution and allowed to bind to antigen-coated wells overnight at 4°C. Plates were washed 3 times and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin at 37°C for 1 hour. Plates were washed and incubated with TMB substrate for 20 minutes before measurement of colour development at A_{450}.

All vaccinia-inoculated mice responded to the vaccination by the detection of immunoglobulin to vaccinia virus in serum (data not shown). Immunoassay to measure TC-83 antibody failed to detect anti-VEE IgG in WR100 samples. WR103 samples contained a detectable level of anti-VEE antibody although this was substantially lower than the amount found in serum from mice vaccinated with TC-83 (Figure 4).

Neutralising antibody was measured by a plaque reduction test. Serum (10μl) was incubated with TC-83 (50μl) and maintenance medium (140 μl) for 1 hour at room temperature. Maintenance medium (800μl) was added and the suspension was used to infect confluent monolayers of BHK-21 cells grown in 6-well plates. Plates were incubated at 37°C for 3 days. A 50% reduction in the number of plaques per well, compared to control wells, was indicative of the presence of neutralising antibody.

Neutralising antibody to TC-83 was found in serum from mice vaccinated with TC-83 but was not detected in serum from mice vaccinated with WR100 or WR103 (data not shown). Although neutralising antibody is usually found in mice which are protected against VEE challenge, protection has previously been reported in the absence of detectable neutralising antibody (Kinney et al, 1988a, Journal of Virology 62, 4697-4702).

Claims

1. A vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine comprising a vector which includes a sequence which encodes an attenuated form of said virus which is capable of producing a protective immune response, wherein the said sequence is such that the amino acid at position 7 in the E2 protein of VEE is lysine.

2. A vaccine according to claim 1 wherein the attenuated form of said virus comprises a derivative of the TC-83 construct.

3. A vaccine according to claim 2 wherein the vector comprises a virus vector.

4. A vaccine according to claim 3 wherein the virus is selected from an attenuated virus

5. A vaccine according to claim 3 or claim 4 wherein the virus is selected from vaccinia, adenovirus, HSV, BCG or BCC.

6. A vaccine according to claim 5 which comprises an attenuated vaccinia virus.

7. A vaccine according to claim 6 wherein expression of the said attenuated VEE virus is under the control of a synthetic 7.5K vaccinia promoter which has been subject to mutation which increases the level of VEE virus protein production as compared to the wild-type 7.5K promoter.

8. A vaccine according to claim 7 wherein the said 7.5K promoter comprises a sequence selected from

TAAAAATTGAAAATACATTCTAATTTATTGCAC (SEQ ID No 2)

or

TAAAAATTGAAAATATATTCTAATTTATTGCAC (SEQ ID No 3).

9. A vaccine according to any one of the preceding claims which comprises a vector which includes a nucleotide sequence which encodes a further immunogenic peptide, and is able to express said sequence when administered to a mammal.

10. A vaccine according to any one of the preceding claims which further comprises a cytokine or an active fragement or variant thereof, or a vector which comprises a nucleotide sequence which encodes a cytokine or an active fragment or variant thereof.

11. A vaccine according to claim 10 which comprises a vector which comprises a nucleotide sequence which encodes a cytokine or an active fragment or variant thereof.

12. A vaccine according to claim 10 or claim 11 wherein the cytokine is an interleukin.

13. A vaccine according to claim 10 wherein the interleukin is selected from human IL-2 or human IL-6.

14. A vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine comprising a vaccinia virus vector which encodes an attenuated form of the VEE virus or a variant or fragment thereof which is capable of producing a protective immune response against VEE virus, expression of the said attenuated VEE virus being under the control of a synthetic 7.5K vaccinia promoter which has been subject to mutation which increases the level of VEE virus protein production as compared to the wild-type 7.5K promoter

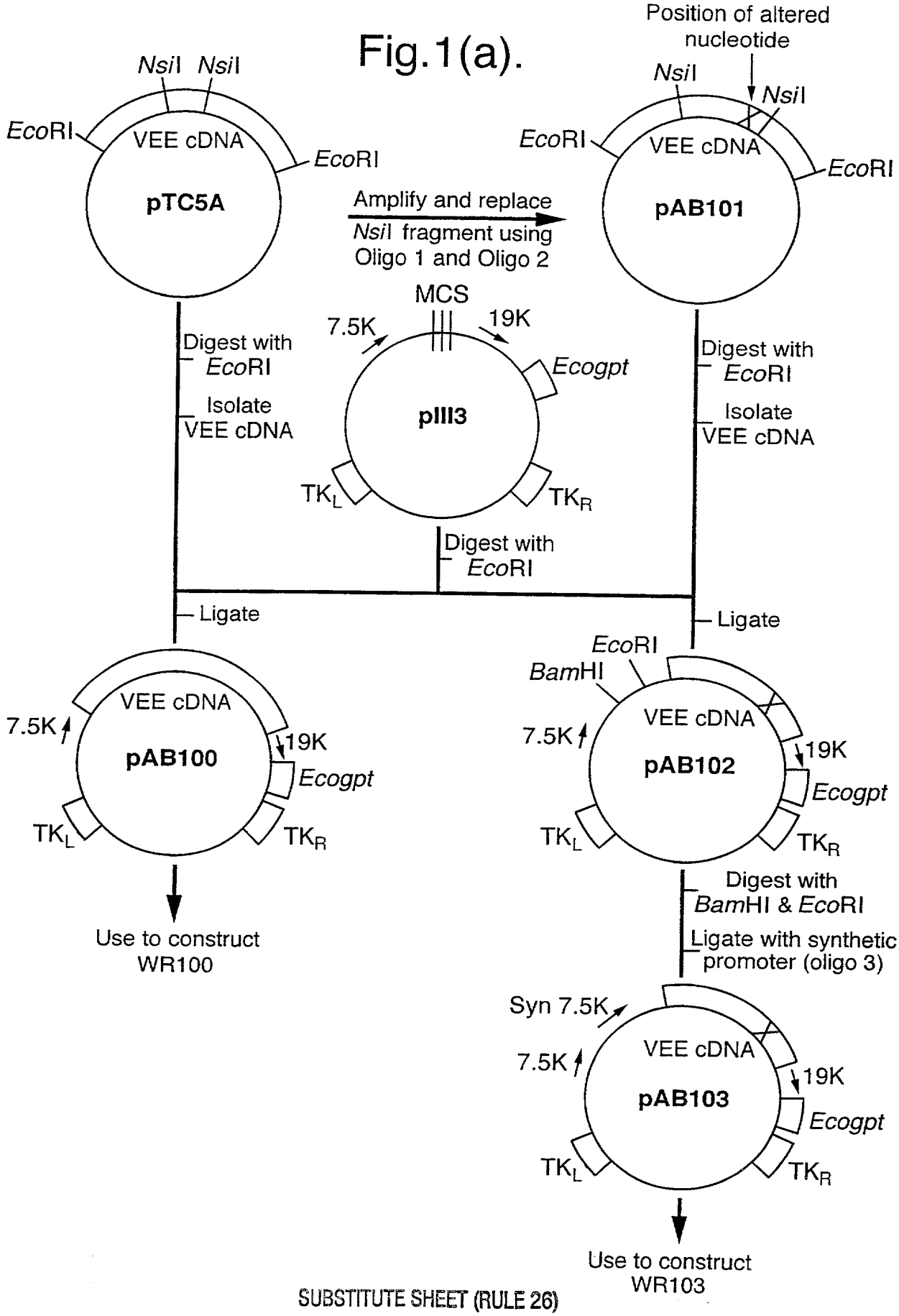
15. A pharmaceutical composition comprising a vaccine as defined in any one of the preceding claims and a pharmaceutically acceptable carrier or excipient.

16. A method for producing a protective immune response against VEE virus in a mammal, which method comprises administering to said mammal, a vaccine according to any one of claims 1 to 14.

17. A method according to claim 16 wherein the mammal is either a human or a horse.

18. A multivalent vaccine comprising a vaccine according to any one of claims 1 to 14 and a further vaccine.

Fig.1(a).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7.5K
19K
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Ligate
Ligate
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19K
Ecogpt
pAB100
TKL
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Use to construct WR100
EcoRI
BamHI
VEE cDNA
7.5K
19K
Ecogpt
pAB102
TKL
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Digest with BamHI & EcoRI
Ligate with synthetic promoter (oligo 3)
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Ecogpt
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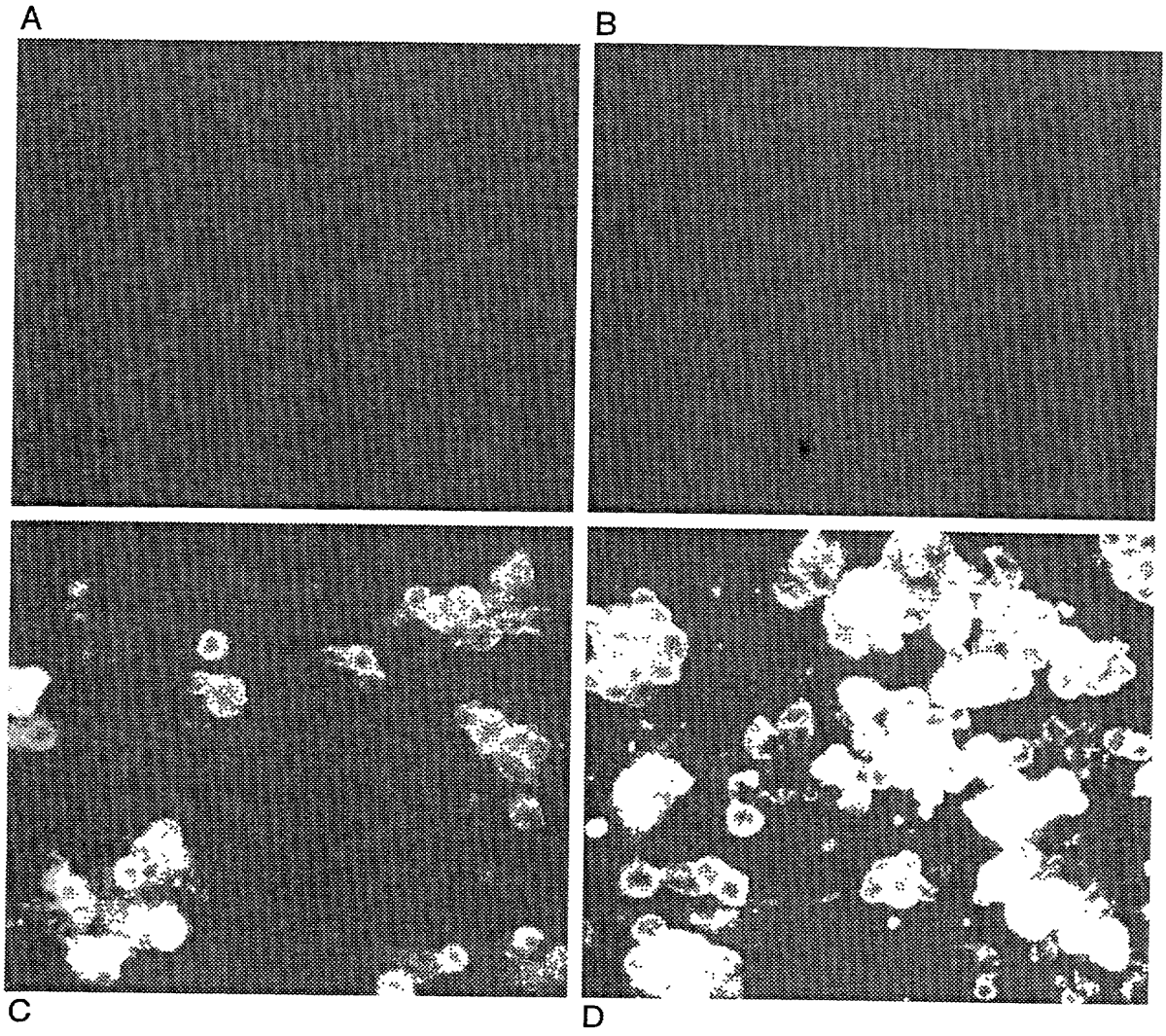
Use to construct WR103

Fig.2.



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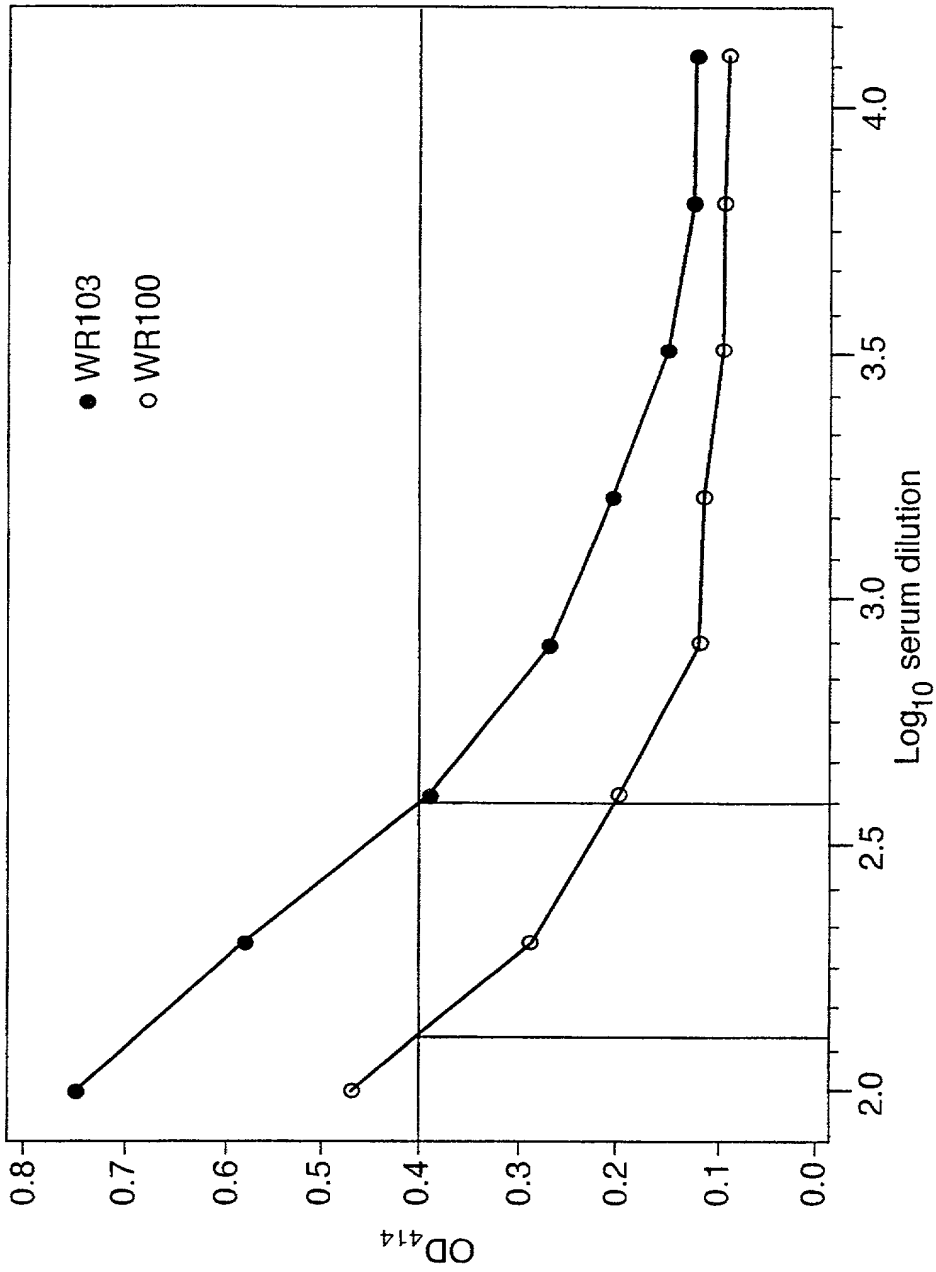
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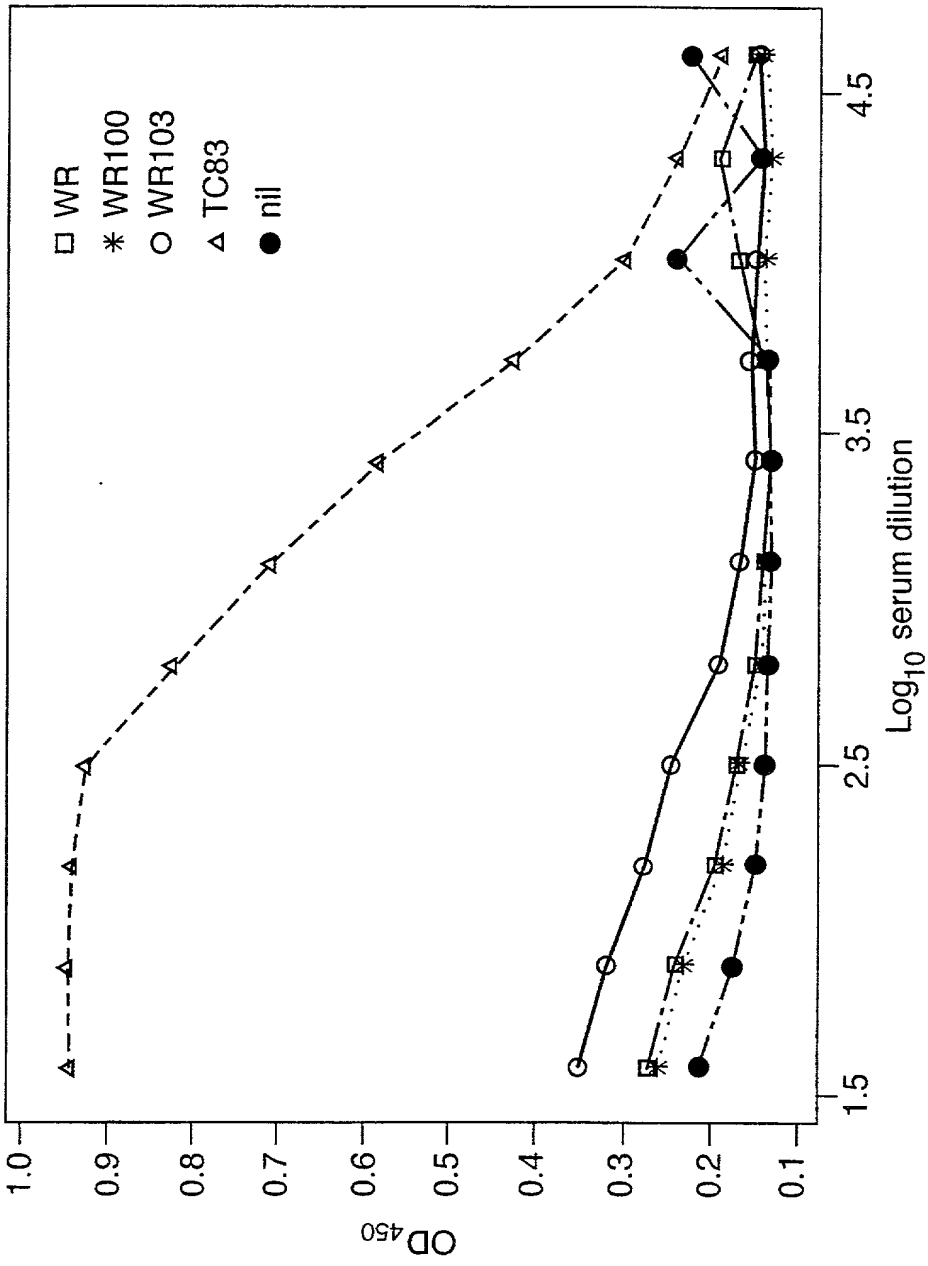
Log_{10} serum dilution

Fig.4.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1.5
2.5
3.5
4.5
Log10 serum dilution

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As below named inventor, I hereby declare that my residence. Post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled.

RECOMBINANT VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE.

The specification of which (check applicable box(s)):

[ ] is attached hereto.

[ ] was filed on _____

as U.S. Application Serial No._____

[X] was filed as PCT international application No. PCT/GB99/01387 filed 5 May 1999

and (if applicable to U.S. or PCT Application) was amended on

_____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. 1.56(A). I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s): Application Number	**County**	**Day/Month/Year Filed**
9811433.3	**GB**	**29 May 1998**

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above and below, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56(a) which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S. /PCT Applications(s):	**Day/Month/Year Filed**	**Status:**
PCT/GB99/01387	**5 May 1999**	**Pending**

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C. 8th Floor, 1100 North Glebe Road, Arlington, Virginia 22201-4714 Telephone number (703) 816-4000 to who all communications are to be directed).** And the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C Mitchard, 29009;; Duane M Byers, 33363; Paul J. Henon, 33626; Jeffry H. Nelson, 30481; John R. Lustova, 33149; H. Warren Burnan, Jr., 29366; Thomas E. Byrne, 32205; Mary J Wilson 32955; J Scott Davidson 33489

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Inventors Signature Alice Bennett Date 13/11/00

Inventors Name (typed)

Alice	M	BENNETT	GB
First	Middle Initial	**Family name**	Citizenship

Residence (city) Salisbury GBN (State/Foreign Country) GB

Post Office Address CBD Porton Down, Salisbury, Wiltshire. Zip Code SP4 0JQ

Inventors Signature _____ Date _____

Inventors Name (typed)

First	Middle Initial	**Family Name**	Citizenship

Residence (City) _____ State/Foreign Country) _____

Post Office Address, _____ Zip Code _____

Inventors Signature _____ Date _____

Inventors Name (typed) _____

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